Discovery of *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev in western Turkey (Anatolia)

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ABSTRACT

Additional analyses of trnS-trnG and nrDNA from herbarium specimens from Europe revealed the presence of *J. sabina* var. *balkanensis* in western Turkey near Izmir and expands the range previously known only from Bulgaria and adjacent mountains in Greece. A more detailed map of the taxon's distribution is presented. Published on-line www.phytologia.org *Phytologia 99(1): 22-31 (Jan 19, 2017)*. ISSN 030319430.

KEY WORDS: *Juniperus sabina var. balkanensis, J. sabina*, distribution, nrDNA, trnS-trnG, chloroplast capture.

Recently, Adams et al. (2016) reported on the capture of *J. thurifera* (or an ancestor) chloroplast by *J. sabina* var. *balkanensis*. Chloroplast capture has been rarely reported in conifers. In *Pinus* and other conifers, Hipkins et al. (1994) concluded that "past hybridization and associated 'chloroplast capture' can confuse the phylogenies of conifers." Bouille et al. (2011) found significant topological differences in phylogenetic trees based on cpDNA (vs. mtDNA sequences) in *Picea* that suggested organelle capture.

In *Juniperus*, Terry et al. (2000) suggested that chloroplast capture was involved in the distribution of cp haplotypes in *J. osteosperma* in western North America. More recently, Adams (2015a, b) found widespread hybridization and introgression between *J. maritima* and *J. scopulorum* in the Pacific northwest, with introgression from *J. maritima* into *J. scopulorum* eastward into Montana. The disparity between cpDNA and nuclear markers (nrDNA and maldehy) suggested that cp capture had occurred.

The *Juniperus* of section *Sabina*, of the eastern hemisphere, can be divided into two groups based on the number of seeds per female cone (often called berries) and female cone shape. The single seed/cone (single-seeded) *Juniperus* of the eastern hemisphere have cones that are ovoid with a noticeable pointed tip, whereas the multi-seeded *Juniperus* are generally globose and often have an

irregular surface (Adams 2014). *Juniperus sabina* L. is a smooth leaf-margined, multi-seeded juniper of the eastern hemisphere. It is very widely distributed from Spain through Europe to Kazakhstan, western China, Mongolia and Siberia (Fig. 1). *Juniperus sabina* has a range that is discontinuous between Europe and central Asia; the species is generally a shrub less than 1 m tall and ranges up to 1-2 m wide. But in the Sierra Nevada of Spain, it forms a horizontal shrub.

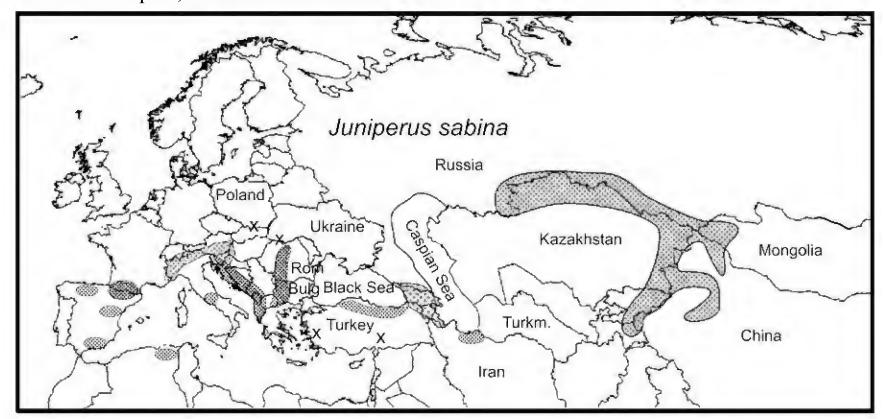


Fig. 1. Distribution (shaded areas) of *J. sabina*. x = outlying populations of*J. sabina*.

Adams et al. (2016) showed that nrDNA (ITS) did not resolve *J. sabina* populations due to the lack of sequence variation.

However, their analyses (Adams et al., 2016) of cp DNA (petN-psbM, trnSG, trnDt, trnLF) revealed that *J. sabina* contained two kinds of cpDNA: typical *J. sabina* and *J. sabina* var. *balkanensis* cpDNA in a clade with *J. thurifera* (Fig. 2).

It might be noted that *J. sabina* from Kazakhstan and Xinjiang form a clade (Fig. 2). The use of four cp regions resulted in a clade of the junipers from the western hemisphere (box, Fig. 2).

In order to investigate the amount of divergence of the 'balkanensis' chloroplast from that of present day *J. thurifera*, a minimum spanning network was computed using both SNPs and indels, herein called mutations. This analysis found 52 mutations within the set: *J. sabina (sensu stricto)*, *J. sabina J. s.* var. *balkanensis* and *J. thurifera*. The minimum spanning network (Fig. 3) shows that all the 'balkanensis' plants differ by only 6-8 mutations from *J. thurifera* chloroplast. However, the nearest link connecting 'balkanensis' to *J. sabina (sensu stricto)* is 36 mutations!

Notice (Fig. 3) that Azerbaijan/ Mongolia accessions group with Kazakhstan/ Xinjiang and this group differs by 7 mutations from the Europe/ Algeria group. This suggests that *J. sabina* in central Asia may be a different variety of *J. sabina*. That needs to be examined in more detail (in progress).

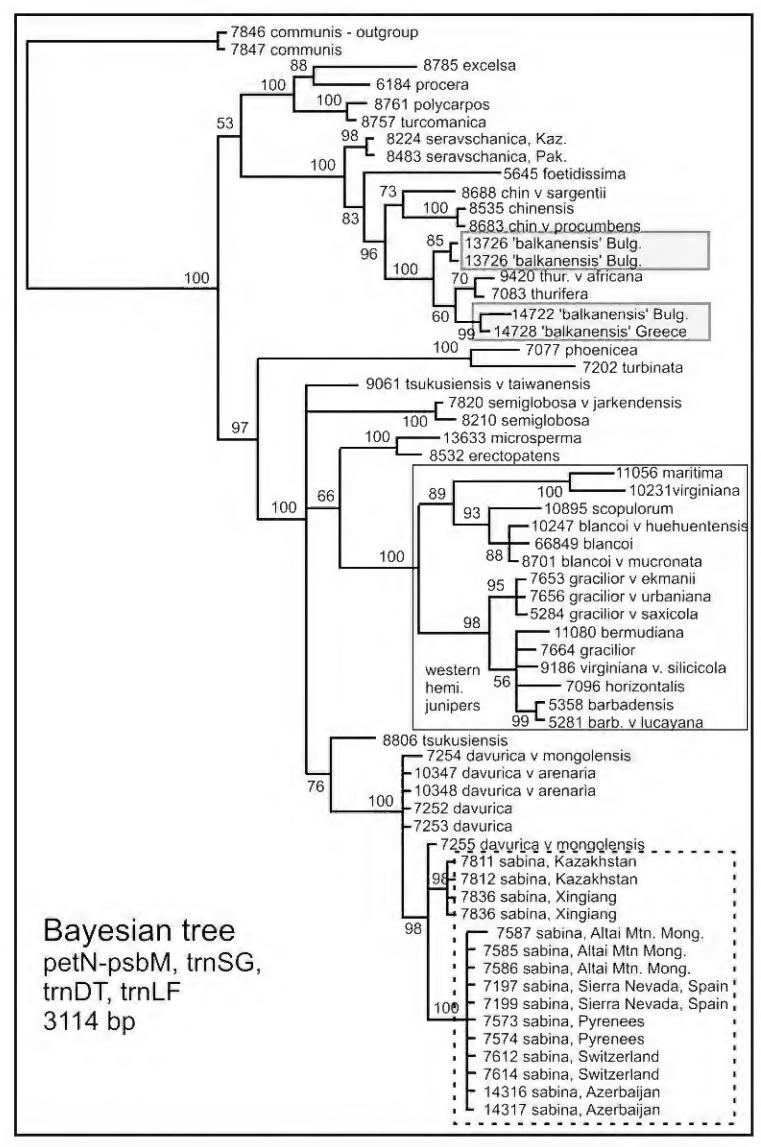


Figure 2. Bayesian analysis based on four cp regions (adapted from Adams et al., 2016).

Adams et al. (2016) concluded that *J. sabina* var. *balkanensis* captured the chloroplast of an ancestor of the *thurifera* lineage during an ancient hybridization event at a time when species distributions overlapped. Because var. *balkanensis* has morphology almost identical to *J. sabina* (*sensu stricto*), this hybridization event was likely followed by successive backcrosses to *J. sabina* after the

hybridization event, resulting in a nuclear genome, including morphology, that is nearly identical to *J. sabina* (*sensu stricto*). In fact, Adams et al., 2016 found in the nrDNA analysis that *J. s.* var *balkanensis* was clearly interspersed in a clade with other *J. sabina*. So it is not surprising that a comparison of the morphology of *J. sabina and J. s.* var. *balkanensis*, has, to date, revealed only a few quantitative differences (Adams et al. 2016, Table 1).

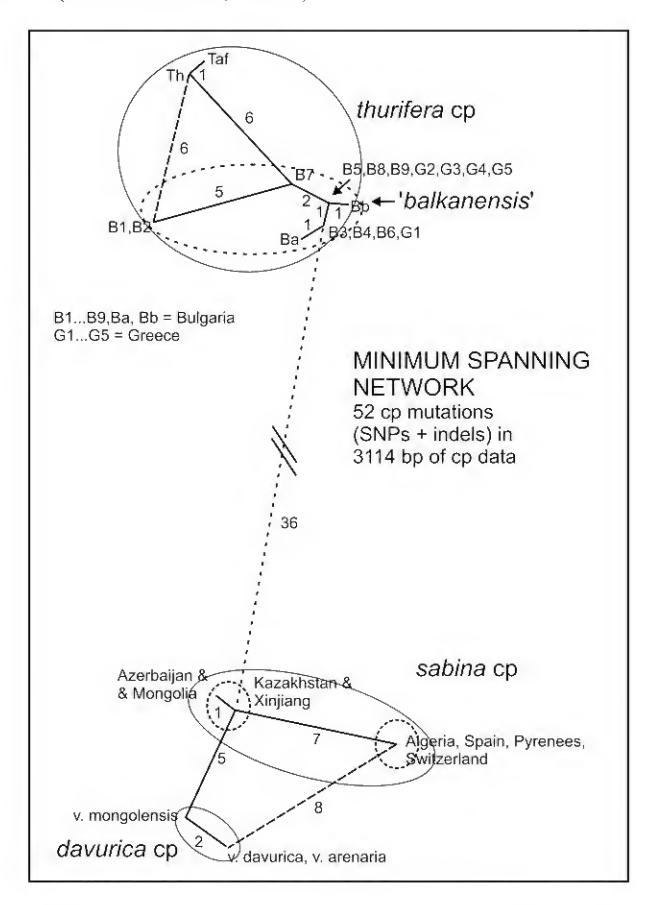


Figure 3. Minimum spanning network based on 52 mutations (SNPs + indels) in 4 cp markers (3114 bp). The numbers next to the lines are the number of mutations for that link. The dotted line connects the *thurifera* cp taxa to the *sabina* cp taxa by 36 mutations. The dashed line is the second nearest neighbor of *J. sabina* to *J. davurica* cp type. (8 mutations).

Juniperus sabina var. balkanensis is known only from sloping rocky limestone, at 1240 - 1630m, in the mountains of Bulgaria and northern Greece (Fig. 4). Adams et al. (2016) postulated that it may occur northward into Romania, westward into Macedonia and/ or eastward into northern Turkey.

The purpose of the present paper is to report on a broader sampling of *J. sabina* from herbarium specimens to more precisely determine the distribution of *J. sabina* var. *balkanensis*.



Fig. 4. Habit and habitat of *J. s.* var. *balkanensis* in the eastern Rhodopes mountains, Bulgaria. *Juniperus communis*, columnar trees, are in the background.

MATERIAL AND METHODS

Specimens used in this study (species, popn. id., location, collection numbers): *J. chinensis*, CH, Lanzhou, Gansu, China, *Adams 6765-6767*; *J. sabina*: (SN), Sierra Nevada, Spain, *Adams 7197*, *7199*, *7200*; (PY), Pyrenees Mtns., Spain/ France border, *Adams 7573-7577*; (SW), Switzerland, *Adams 7611*, *7612*, *7614*, *7615*; TS, Tian Shan Mtns., Xinjiang, China, *Adams 7836-7838*; Mongolia, Altai Mtns., *Adams 7585-7587*; Kazakhstan, Paniflor, *Adams 7811-7812*; Azerbaijan: *Adams 14316-14320*; *J. davurica* (DV), 15 km se Ulan Bator, Mongolia, *Adams 7252*, *7253*, *7601*; *J. davurica* var. *arenaria*

J. davurica (DV), 15 km se Ulan Bator, Mongolia, Adams 7252, 7253, 7601; J. davurica var. arenaria (AR) sand dunes, Lake Qinghai, Qinghai, China, Adams 10347-10352; river bank, Gansu, J-Q. Liu and Adams 10354-10356; J. davurica var. mongolensis (MS) sand dunes, 80 km sw Ulan Bator, Mongolia, Adams 7254-7256;

Collections of taxon with non-J. sabina cpDNA in Adams, Schwarzbach and Tashev (2016): (acronyms used in Fig. 7)

Bulgaria and Greece

B1-B5 Eastern Rhodopes, Bulgaria, *Adams 13725-13729 (A. Tashev 2012-1-5)*;

B6 Central Stara Plania, Sokolna reserve, Bulgaria, Adams 14721 (A. Tashev 2015 Balkan 1;

B7-B9, Ba, Bb Rila Mountain, Bulgaria, Adams 14722-14726 (A. Tashev 2015 Rila 1.1-1.3, 2.1-2.2);

G1-G5 Mt. Tsena, Greece, Adams 14727-14731 (A. Tashev 2015 So. 1-5 Tsena);

Samples new for this study: (with Lab Acc. ID = Adams xxxxx)

Austria

14872 Austria, Alps, Ötztal, Zwiselstein, N 46.935°, E11.039°, 1650-1700m alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.001, KOR 51592, female

14873 Austria, Alps, Ötztal, Below Sőlden, N 46.994°, E11.012°, 1300 alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.005, KOR 51596, male

14874 Austria, Alps, Ötztal, Below Sőlden, N 46.994°, E11.012°, 1300 alt., leg. K. Boratyńska, A.Boratyński, 2015, 15.005, KOR 51595, female

France

14863 France, Alps de Dauphiné, St. Crépin, N 44.71°, E 6.61°, ca 1000m alt, leg. A. Boratyński, K. Boratyńska 2003, 03.19.116, KOR 43778, female

Italy

14870 Italy, Alps, Val d'Aosta, Introd, Les Combes, N 45.689°, E 7.166°, 1250 m alt. Lag. K. Boratyńska, A. Boratyński, 15.014. KOR 51590, female

14871 Italy, Alps, Val d'Aosta. Introd, Les Combes, N 45.689°, E 7.166°, 1250 m alt. Lag. K. Boratyńska, A. Boratyński, 15.013. KOR 51589, male

Poland

14858 Poland, Carpathians, Pieniny National Park, Facimiech, N 49.40°, E 20.43°, ca 600m alt. From specimen propagated vegetatively about 2005 and planted in dendrological garden of Forest Botany Chair, Forest Faculty, Poznań University of Life Sciences

Russia

14865 Russia, Altay, Aktru Valley, SWW of Bielucha Mt., ca. N 49.80°, E 86.40°, 2500m alt., leg. Faltynowicz W., 2010. KOR 4796, female.

Spain

14860 Spain, Cuenca, Serraña de Cuenca, between Tragacete and La Cueva (Vega de Cordorno), N 40.433°, W 1.905°, ca 1450 m alt., lg. Boratyńska K., Boratyński A., 2006, SP.06.026, KOR 44733, female

14862 Spain, Teruel, Puerto de Cabigordo near Cedrillas E of Teruel, N 40.41°, W0.95°, ca 1500m alt., Leg. A. Boratyński, K. Boratyńska, HS_01.03.17, KOR 43212,

14864 Spain, Sierra Nevada, Veleta Mt., above Alberque Universitario, N 37.09°, W 3.38°, ca 2500m alt., leg. A. Boratyński 1991, KOR 25299

14866 Spain, Sierra Nevada, Monte Ahí de Cara, N 37.13°, W3.43°, 1900-2000m alt., leg. A.Boratyński, Ja. Didukh., D.Tomaszewski, Z. Boratyński, KOR 46220, female

14869 Spain, Leon, Los Barios de Luna, N 42.88°, W 5.87°, 1150-1200m alt., leg. K.Boratyńska, A. Boratyński, 2015, KOR 51542, female

14875 Spain, Sierra de Albarracin, S of Brochales, N 40.50°, W 1.57°, ca 1600m alt., leg. A. Boratyński, K. Boratyńska, 2006. female

14876 Spain, Aragon, Moncayo, N 41.77°, W 1.80°, ca 1900-2000m alt., leg. D. Gomez, 2004, female Switzerland

14867 Switzerland, Alps, Visp, Aussenberg, N 46.31°, E 7.87°, ca 950-1000m alt., leg. K.Boratyńska, A.Boratyński, 2015, 15.016, KOR 51570, female

14868 Switzerland, Alps, Visp, Aussenberg, N 46.31°, E 7.87°, ca 950-1000m alt., leg. K.Boratyńska, A.Boratyński, 2015, 15.017, KOR 51581, male

Turkey

14861 Turkey, Manisa. Spil Daği Milli Parki (National Park) (Tas Suret), N38.55°, E 27.42°, ca 1250 m alt., leg. A. Boratyński, K. Boratyńska, 2005, TU 05/55, KOR 44573, female

14934 Turkey, Manisa, Spil Daği Milli Parki (National Park), N38°, 57', E 27° 41', 1024 m., Tuğrul Mataraci 2016-1

14938 Turkey, Gümüshane, Kürtün, Aktas village, Karakaya (Northeast Anatolia), 40° 36' 03" N, 38° 53' 21" E., 2376 m. Coll. A. Kandemir 10745.

Ukraine

14859 Ukraine, Crimea, Chatyr Dag, N 44.773°, E 34.313°, 1100-1200m alt. Lg. A. Boratyński, G. Iszkuło, A. Lewandowski, 2006. UA06.007, KOR 45572

Voucher specimens for all collections are deposited at Baylor University Herbarium (BAYLU) and Herbarium (University of Forestry, Sofia, Bulgaria).

One gram (fresh weight) of the foliage was placed in 20 g of activated silica gel and transported to the lab, thence stored at -20° C until the DNA was extracted. DNA was extracted from juniper leaves by use of a Qiagen mini-plant kit (Qiagen, Valencia, CA) as per manufacturer's instructions. Amplifications were performed in 30 µl reactions using 6 ng of genomic DNA, 1.5 units Epi-Centre Fail-Safe Taq polymerase, 15 µl 2x buffer E (petN, trnD-T, trnL-F, trnS-G) or K (nrDNA) (final concentration: 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 200 µM each dNTP, plus Epi-Centre proprietary

enhancers with 1.5 - 3.5 mM MgCl₂ according to the buffer used) 1.8 μM each primer. See Adams, Bartel and Price (2009) for the ITS and petN-psbM primers utilized. The primers for trnD-trnT, trnL-trnF and trnS-trnG regions have been previously reported (Adams and Kauffmann, 2010). The PCR reaction was subjected to purification by agarose gel electrophoresis. In each case, the band was excised and purified using a Qiagen QIAquick gel extraction kit (Qiagen, Valencia, CA). The gel purified DNA band with the appropriate sequencing primer was sent to McLab Inc. (San Francisco) for sequencing. Sequences for both strands were edited and a consensus sequence was produced using Chromas, version 2.31 (Technelysium Pty Ltd.) or Sequencher v. 5 (genecodes.com). Sequence datasets were analyzed using Geneious v. R7 (Biomatters. Available from http://www.geneious.com/), the MAFFT alignment program. Further analyses utilized the Bayesian analysis software Mr. Bayes v.3.1 (Ronquist and Huelsenbeck 2003). For phylogenetic analyses, appropriate nucleotide substitution models were selected using Modeltest v3.7 (Posada and Crandall 1998) and Akaike's information criterion. Minimum spanning networks were constructed from mutational events (ME) data using PCODNA software (Adams, Bartel and Price, 2009; Adams, 1975; Veldman, 1967).

RESULTS

The results of this study (and the previous, Adams et al., 2016 study) are given in Table 1. The distribution of *J. sabina* var. *balkanensis* and *J. sabina* is shown in Fig. 5. The distribution of *J. thurifera*

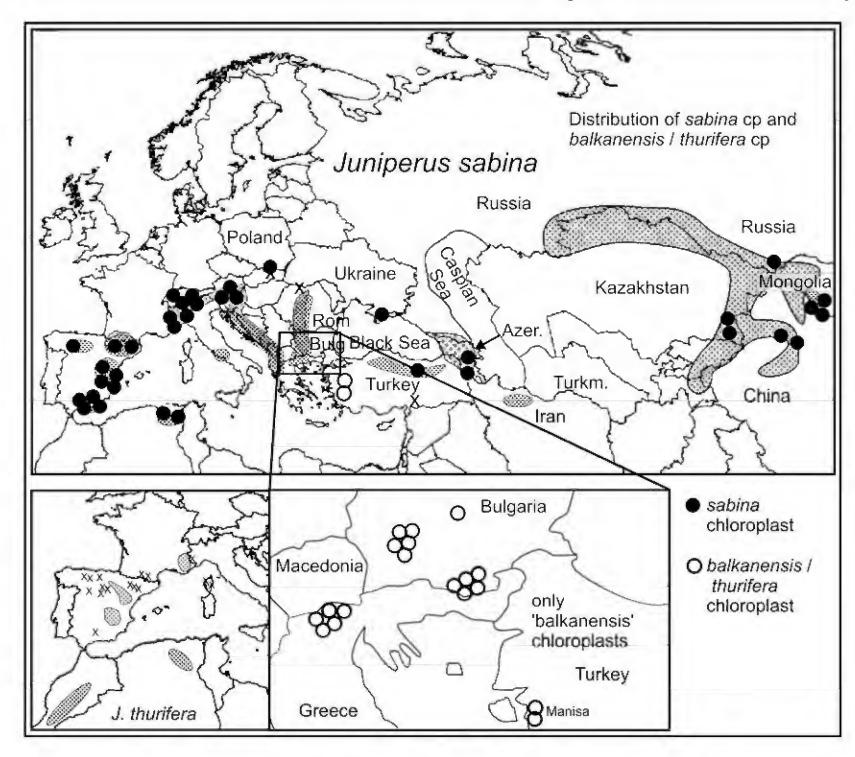


Figure 5. Distribution of *J. sabina* var. *balkanensis* and typical *J. sabina* chloroplast. The present day distributions of *J. thurifera* and var. *africana* (in north Africa) are shown in the insert on the lower left.

is presented in the insert, lower left (Fig. 5). It appears that *J. s.* var. *balkanensis* has a quite restricted range. Additional samples are needed from Romania, Turkey and northwesterly from Albania/Macedonia northwesterly to Slovenia to determine the distribution more precisely.

At present level of understanding, the distributions of *J. s.* var. *balkanensis* and *J. thurifera* do not appear to overlap, negating modern hybridization. However, there were large changes in plant distributions in the Pleistocene and earlier, it seem probable that *J. thurifera*-like ancestors were sympatric with *J. sabina*, and presenting opportunities for chloroplast capture from *J. thurifera*.

ACKNOWLEDGEMENTS

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LITERATURE CITED

- Adams, R. P. 1975. Statistical character weighting and similarity stability. Brittonia 27: 305-316.
- Adams, R. P. 2014. The junipers of the world: The genus Juniperus. 4th ed. Trafford Publ., Victoria, BC.
- Adams, R. P. 2015a. Allopatric hybridization and introgression between *Juniperus maritima* R. P. Adams and *J. scopulorum* Sarg.: Evidence from nuclear and cpDNA and leaf terpenoids. Phytologia 97: 55-66.
- Adams, R. P. 2015b. Allopatric hybridization and introgression between *Juniperus maritima* R. P. Adams and *J. scopulorum* Sarg. II. Additional Evidence from nuclear and cpDNA genes in Montana, Wyoming, Idaho and Utah. Phytologia 97: 189-199.
- Adams, R. P., J. A. Bartel and R. A. Price. 2009. A new genus, *Hesperocyparis*, for the cypresses of the new world. Phytologia 91: 160-185.
- Adams, R. P. and M. E. Kauffmann. 2010. Geographic variation in nrDNA and cp DNA of *Juniperus californica*, *J. grandis*, *J. occidentalis* and *J. osteosperma* (Cupressaceae). Phytologia 92: 266-276.
- Adams, R., A. E. Schwarzbach and A. N. Tashev. 2016. Chloroplast capture in *Juniperus sabina* var. *balkanensis* R. P. Adams and A. N. Tashev, from the Balkan peninsula: A new variety with a history of hybridization with *J. thurifera*. Phytologia 98: 100-111.
- Bouille, M., S. Senneville and J. Bousquet. 2011. Discordant mtDNA and cpDNA phylogenies indicate geographic speciation and reticulation as driving factors for the diversification of the genus *Picea*. Tree Genetics & Genomes 7: 469-484.
- Hipkins, V. D., K. V. Krutovskii and S. H. Strauss. 1994. Organelle genomes in conifers: structure, evolution, and diversity. Forest Genetics 1: 179-189.
- Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14: 817-818.
- Ronquist, F. and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574.
- Terry, R. C., R. S. Nowak and R. J. Tausch. 2000. Genetic variation in chloroplast and nuclear ribosomal DNA in Utah juniper (*Juniperus osteosperma*, Cupressaceae): evidence of interspecific gene flow. Am. J. Bot. 87: 250-258.
- Veldman, D. J., 1967. Fortran programming for the behavioral sciences. Holt, Rinehart and Winston Publ., NY.

Table 1. Classification of *J. sabina* specimens based on trnS-trnG (plus petN-psbM, trnDT, trnLF) and nrDNA (ITS).

	trnSG (cp genome)	nrDNA
Lab Acc. #, Location	classification	classification
13725 Bulgaria, eastern Rhodopes	v. balkanensis	v. sabina
13726 Bulgaria, eastern Rhodopes	v. balkanensis	v. sabina
13727 Bulgaria, eastern Rhodopes	v. balkanensis	v. sabina
13728 Bulgaria, eastern Rhodopes	v. balkanensis	v. <i>sabina</i>
13729 Bulgaria, eastern Rhodopes	v. balkanensis	v. sabina
14721 Bulgaria, Sokolna reserve	v. balkanensis	v. sabina
14722 Bulgaria, Rila Mtn.	v. balkanensis	v. sabina
14723 Bulgaria, Rila Mtn.	v. balkanensis	v <i>sabina</i>
14724 Bulgaria, Rila Mtn.	v. balkanensis	v. sabina
14725 Bulgaria, Rila Mtn.	v. balkanensis	v. sabina
14726 Bulgaria, Rila Mtn.	v. balkanensis	v. sabina
14727 Greece, Tsena Mt.	v. balkanensis	v. sabina
14728 Greece, Tsena Mt.	v. balkanensis	v. <i>sabina</i>
14729 Greece, Tsena Mt.	v. balkanensis	v. sabina
14730 Greece, Tsena Mt.	v. balkanensis	v. <i>sabina</i>
14731 Greece., Tsena Mt.	v. balkanensis	v <i>sabina</i>
14934 w Turkey, Spil Daği Milli Parki	v. balkanensis	v. sabina
14861 w Turkey, Spil Daği Milli Parki	v. balkanensis	v. sabina
13167 Algeria	v. sabina	v. sabina
13168 Algeria	v. sabina	v. sabina
14872 Austria, Otztal, Zwiselstein	v. sabina	v. sabina
14873 Austria, Ötztal, Below Sőlden,	v. sabina	v. <i>sabina</i>
14874 Austria, Ötztal, Below Sőlden,	v. sabina	v. sabina
14316 Azerbaijan	v. sabina	v. sabina
14317 Azerbaijan	v. sabina	v. sabina
7836 China, Heaven Lake, Xinjiang	v. sabina	v. sabina
7837 China, Heaven Lake, Xinjiang	v. sabina	v. sabina
14863 France, Alps de Dauphine	v. sabina	v. sabina
7573 France, Pyrennes Mtns	v. sabina	v. sabina
7574 France, Pyrennes Mtns	v. sabina	v. sabina
14870 Italy, Val d'Aosta, Alps	v. sabina	v. sabina
14871 Italy, Val d'Aosta, Alps	v. sabina	v. sabina
7811 Kazakhstan, Paniflor	v. sabina	v. sabina
7812 Kazakhstan, Paniflor	v. sabina	v. <i>sabîna</i>
7585 Mongolia, Altair Mtns	v. sabina	v. sabina
7586 Mongolia, Altair Mtns	v. sabina	v <i>sabina</i>
7587 Mongolia, Altair Mtns	v. sabina	v. sabina
14858 Poland, Pieniny N.P.,	v. sabina	v. sabina
14865 Russia, Altay Mtn.	v. sabina	v. sabina
7197 Spain, Sierra Neyada	v. sabina	v. sabina
7199 Spain, Sierra Nevada	v. sabina	v. sabina
14860 Spain, Serrana de Cuenca	v. sabina	v. <i>sabina</i>
14862 Spain, Teruel	v. sabina	v. sabina
14864 Spain, Sierra Nevada	v. sabina	v. <i>sabina</i>
14866 Spain, Sierra Nevada	v. sabina	v. sabina
14869 Spain, Los Barios de Luna	v. sabina	v. <i>sabina</i>
14875 Spain, Sierra de Albarracin	v. sabina	v. sabina
14876 Spain, Aragon, Moncayo	v. <i>sabina</i>	v. sabina

7611 Switzerland, Alps	v. sabina	v. sabina
7612 Switzerland, Alps	v. sabina	v. sabina
7614 Switzerland, Alps	v. sabina	v. sabina
14867 Switzerland, Aussenberg	v. sabina	v. sabina
14868 Switzerland, Aussenberg	v. sabina	v. sabina
14938 northeast Turkey	v. sabina	v. sabina
14859 Ukraine, Crimea, Chatry Dag	v. sabina	v. sabina